PLATELET PLASMA MEMBRANE GLYCOPROTEINS

Identification of a Proteolytic Substrate for Thrombin*

David R. Phillips+ and Patricia Poh Agin

Department of Biochemistry
St. Jude Children's Research Hospital
332 North Lauderdale
Memphis, Tennessee 38101

Received March 7,1977

SUMMARY: The surface glycoproteins of the platelet plasma membrane were labeled by oxidation with galactose oxidase followed by reduction with (^{3}H)-sodium borohydride. Of the glycoproteins labeled, only glycoprotein V (apparent molecular weight of 89,000) was decreased as a result of thrombin action. The affected glycoprotein appeared to be completely removed at a concentration of 1 U thrombin per 10^9 platelets. A soluble glycopeptide hydrolytic product with an apparent molecular weight of 70,000 was released into solution.

INTRODUCTION

The mode of interaction of thrombin with cells is developing into a question with broad biological interest. It has been known for some time that this proteolytic enzyme is one of the most important - and most potent - stimulators of platelets during hemostasis and thrombosis. More recently, it has been shown that thrombin is also a potent mitogenic agent for mammalian cells (1). In all cases, these activities of thrombin appear to be dependent upon its proteolytic activity since blocking of active site serine inhibits activity (1, 2). Further, serine proteases with related specificity, e.g., trypsin, mimic these activities of thrombin (3, 4).

Studies of the mechanism of proteolytic stimulation require identification of the proteolytic receptor site. To date, the reports concerning these receptor sites on mammalian cells grown in culture have been con-

* Supported in part by grant HL-15616 from the U.S. Public Health Service.

+ Recipient of a Research Career Development Award (HL-0080) from the U.S. Public Health Service.

ISSN 0006-291X

flicting (5-7). Likewise, the thrombin proteolytic receptor on platelets has been the subject of much debate (8-11). In a previous report (12), we showed that one of the glycoproteins iodinated by lactoperoxidase on intact platelets was altered by thrombin treatment. However, only 30% of this component could be removed even at the high thrombin concentration used. In addition, hydrolysis during removal was not demonstrated since no hydrolytic product was identified. Recently, we showed (13) that there are more glycoproteins in the platelet plasma membrane than previously recognized. Using improved electrophoretic techniques, we were able to resolve the membrane glycoproteins. As reported herein, we are now able to separate the glycoprotein proteolytic substrate for thrombin from other membrane glycoproteins and show that thrombin removes this substrate from the membrane surface. The glycopeptide hydrolytic fragment was also identified.

MATERIALS AND METHODS

Platelets were isolated from fresh human blood (14) and labeled by the galactose oxidase procedure (15). Briefly, 10^9 washed platelets were suspended in 1 ml Tyrode's solution and incubated for 15 min with 10 units* *V. cholerae* neuraminidase (Schwarz Mann Biochemicals), for 5 min with 3.25 units* galactose oxidase (Worthington Biochemicals, further purified by the method of Hatton and Regoeczi (16) to a specific activity of 830 units mg^{-1} protein), and finally for 5 min with 0.5 mCi $Na[^3H]BH_4$ (Research Products International, 15 Ci $millimole^{-1}$). All incubations were at 37° and the platelets were washed once between each incubation.

The labeled platelets were suspended in 1 ml of a buffer containing 0.15 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 7.6, and treated with 1 N.I.H. unit human thrombin at 37° for 15 min. The thrombin used (a gift from Dr. J. W. Fenton) had a specific activity of 2,800 N.I.H. units mg^{-1} protein; one N.I.H. unit equals approximately 10^{-11} moles of active enzyme. The platelets were removed from solution by centrifugation, washed once, and solubilized in sodium dodecyl sulfate (17). The platelet polypeptides (40 µg protein) were separated by electrophoresis according to Laemmli (18) and stained with Coomassie blue (17). The tritium distribution in the dried gels was obtained by fluorography according to the method of Bonner and Laskey (19).

Serotonin secretion was determined by the radioactive method previously described (20, 21). Washed platelets (2×10^9) were suspended in 5 ml of a buffered salt solution containing 130 mM NaCl, 3 mM KCl, 0.06 mM $CaCl_2$, 0.02 mM $MgCl_2$, 26 mM N-tris(hydroxyl methyl)methyl 2-amino ethane sulfonic acid (Sigma Chemical Co.) and 25 mM glucose, pH 6.8. We then added [^{14}C] serotonin (Amersham-Searle, 55 Ci $mole^{-1}$) to a final concentration of 0.85 µM and incubated the solution for 1 hr at 18° with occasional agitation. After two washes,

* The definitions of galactose oxidase and neuraminidase units were as described by the suppliers.

TABLE I

Effect of Galactose Oxidase Labeling on Thrombin-Induced Secretion

Platelet Treatment	% ^{14}C-Serotonin Secreted	
	Labeled Platelets	Control Platelets
Labeling Incubations:		
Neuraminidase	0	---
Galactose Oxidase	2	---
Na[^{3}H]BH$_4$	3	---
Thrombin Incubations*:		
Control (minus thrombin)	8	7
Thrombin treated (1 unit ml^{-1} thrombin)	46	48

*Determined after 5 min incubations.

the platelets were suspended to a concentration of 10^9 ml^{-1} in the tris-saline-EDTA buffer. The release reaction was initiated by adding thrombin to 0.5 ml samples and incubating at 37°. At the end of the incubations, the solution was centrifuged for 0.5 min in a Beckman microcentrifuge and the [^{14}C] in an aliquot of the supernatant was counted. Serotonin release was also monitored after each incubation in the labeling procedure.

RESULTS AND DISCUSSION

Our approach to identifying the proteolytic substrate on the membrane surface was to radioactively label the membrane surface glycoproteins and determine which of these were hydrolyzed by thrombin. The labeling method employed oxidation by galactose oxidase followed by reduction with [^{3}H]-sodium borohydride introducing tritium onto the number six carbon of pentultimate galactose residues (15). The labeled platelets were found to aggregate to ADP, collagen and thrombin like unlabeled platelets. In addition, the serotonin stored in platelet granules was not released during labeling but was secreted in response to thrombin (Table 1). It thus appeared that the platelets were not damaged by the labeling process. More platelet glycoproteins were identified by the galactose oxidase labeling procedure

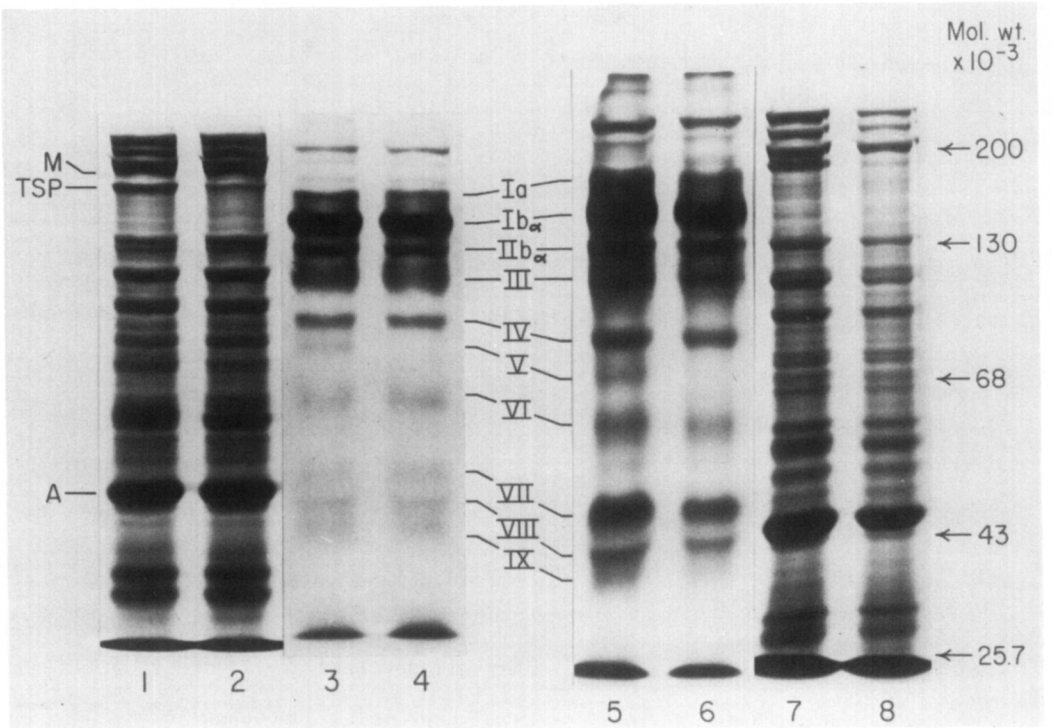

Figure 1 - *Effect of thrombin on platelet membrane glycoprotein V.* Washed platelets from two donors (lanes 1-4 and 5-8, respectively) were labeled by the galactose oxidase/Na[^{3}H]BH_4 procedure, incubated either with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) thrombin and collected by centrifugation. The platelet polypeptides were solubilized in sodium dodecyl sulfate and separated by electrophoresis in 7.5% acrylamide slab gels. The polypeptides in lanes 1, 2, 7 and 8 were stained with Coomassie blue; lanes 3 and 4 are fluorograms of lanes 1 and 2 and show the tritium distribution in the gel; lanes 5 and 6 are fluorograms of lanes 7 and 8. The molecular weights indicated refer to the positions of the molecular weight standards platelet myosin, β-galactosidase, bovine serum albumin, ovalbumin and chymotrypsinogen A, respectively. The gels from the two donors were electrophoresed to different lengths.

(Figure 1, lanes 3 and 5) than have been observed by periodic acid-Schiff staining (17). Glycoproteins V (Mr = 89,000), VI (Mr = 61,500), VII (Mr = 54,000), VIII (Mr = 47,000), and IX (Mr = 44,000) have not been previously described. This labeling method was based on enzyme-catalyzed modifications and presumably modified components only on the membrane's outer surface (15, 22). Indeed, all of the labeled glycoproteins were isolated at a higher

specific activity with the plasma membrane, and trypsin and chymotrypsin hydrolyzed all the labeled membrane glycoproteins of intact platelets, with the exception of glycoprotein IV (D. R. Phillips and P. P. Agin, unpublished results).

Figure 1 also shows the effect of thrombin on labeled platelets from two donors. The Coomassie-stained gels in this figure show that thrombin caused one major change in the polypeptide composition of the platelet; a decrease in the glycoprotein TSP*. This observation has previously been reported on unlabeled platelets (10), and on platelets that had been iodinated by the lactoperoxidase procedure (12). It appears that this glycoprotein is located within platelet granules (17); when secretion is induced by thrombin, TSP is released to the outside of the cell.

Only one of the membrane glycoproteins labeled by the galactose oxidase procedure was affected by thrombin. This glycoprotein, V, showed a marked decrease in concentration. The loss of glycoprotein V appeared to be complete at the ratio of 1 N.I.H. unit of thrombin to 10^9 platelets since no further changes were observed when the thrombin concentration was increased to 10 N.I.H. units/10^9 platelets.

We next wanted to determine whether glycoprotein V was hydrolyzed or released intact from the membrane surface. Figure 2 shows an electrophoretic analysis of the polypeptides in the supernatant fraction after the thrombin-treated platelets were removed from solution by centrifugation. Several polypeptides were observed which were not present in the control (minus thrombin). Most of these were due to polypeptides in the thrombin preparation. One polypeptide (Mr = 64,000) was released from the platelet because of thrombin. Polypeptides are released from intracellular organelles following thrombin stimulation (23), and this polypeptide may be one of them. Alternatively, the Mr = 64,000 polypeptide could be a plasma membrane component not labeled by the galactose oxidase procedures.

* Abbreviations: TSP, thrombin sensitive protein (10); SDS, sodium dodecyl sulfate.

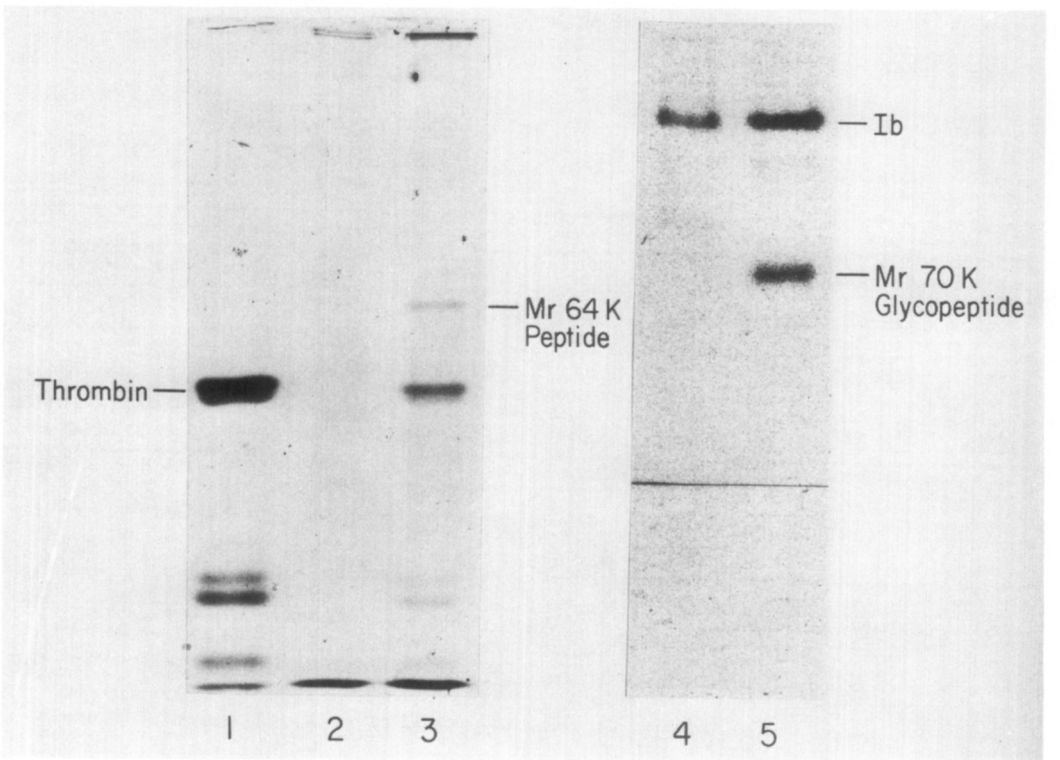

Figure 2 - *Identification of a thrombin-induced glycopeptide hydrolytic product.* Labeled platelets were treated with thrombin and removed from solution by centrifugation. An aliquot (100 µl) of the supernatant was mixed with 50 µl of the SDS containing sample buffer and subjected to electrophoresis in 10% acrylamide slab gels. Lane 1, 5 µg thrombin; lane 2, control supernatant (*minus thrombin*); lane 3, supernatant of thrombin-treated platelets. The polypeptides in lanes 1-3 were stained for protein; lanes 4 and 5 are fluorograms of lanes 2 and 3.

The fluorograms of gels of the soluble fraction in Figure 2 show that one radioactive component (Mr = 70,000) was released from the labeled platelets by the action of thrombin. Since the Mr = 70,000 glycopeptide contained tritium, it appeared to be derived from the plasma membrane. No glycoprotein with this apparent molecular weight was labeled by galactose oxidase treatment of intact platelets, indicating that this was a new molecular species. This glycopeptide was present in the soluble phase only when the amount of platelet-bound glycoprotein V was decreased, indicating that

the Mr = 70,000 glycopeptide was a hydrolytic product of glycoprotein V. This is the first indication that thrombin acts proteolytically towards platelet membrane glycoprotein. The presence of the radioactive glycoprotein with a molecular weight similar to glycoprotein Ib was most likely due to a small amount of platelets in the supernatant.

In previous studies (12), we examined the effect of thrombin on platelets which had been labeled by lactoperoxidase-catalyzed iodination. Using 5% acrylamide gels containing phosphate buffers, we found that one polypeptide was specifically affected by thrombin. This electrophoretic method was not able to resolve the affected glycoprotein from other membrane glycoproteins. In the present study, however, the combination of galactose oxidase/Na[^{3}H]BH_4 labeling and the electrophoretic gel system of Laemmli permitted resolution of the effected glycoprotein, now termed V, from other membrane components. The apparent molecular weight determined in the present study (89,000) differs from our previous value (118,000) and most likely reflects the anomolous electrophoretic mobilities of glycoproteins on different gel systems (24).

Studies from several laboratories have shown the existence of more than one class of thrombin receptors on the platelet membrane surface (25-27). The present results indicate that glycoprotein V may be one of these receptors. Further studies are required, however, to determine if glycoprotein V hydrolysis causes thrombin-induced platelet stimulation.

REFERENCES

1. Chen, L.B. and Buchanan, J.M. (1975) *Proc. Nat. Acad. Sci., 72:* 131-135.
2. Davey, M.G. and Lüscher, E.F. (1967) *Nature (Lond.), 216:* 857-858.
3. Burger, M.M. (1970) *Nature (Lond.), 227:* 171-171.
4. Sefton, B.M. and Rubin, H. (1974) *Nature (Lond.), 227:* 843-845.
5. Hynes, R.O. and Humphryes, K.C. (1974) *J. Cell. Biol., 62:* 438-448.
6. Blumberg, P.M. and Robbins, P.W. (1975) *Cell, 6:* 137-147.
7. Zetter, B.R., Chen, L.B. and Buchanan, J.M. (1976) *Cell, 7:* 407-412.
8. Morse, E.E., Jackson, D.P. and Conley, C.L. (1965) *J. Clin. Invest., 44:* 809-816.
9. Cohen, I., Bohak, I., DeVries, A. and Katchalski, E. (1969) *Europ. J. Biochem., 10:* 388-394.
10. Baenziger, N.L., Brodie, G.N. and Majerus, P.W. (1971) *Proc. Nat. Acad. Sci., U.S., 68:* 240-243.

11. Kisselbach, T.H. and Wagner, R.H. (1966) *Amer. J. Physiol., 211:* 1472-1476.
12. Phillips, D.R. and Agin, P.P. (1974) *Biochim. Biophys. Acta., 352:* 218-227.
13. Phillips, D.R. and Agin, P.P. (in press) *J. Biol. Chem.*
14. Jenkins, C.S.P., Phillips, D.R., Clemetson, K., Meyer, D., Larrieu, M.-J. and Lüscher, E.F. (1976) *J. Clin. Invest., 57:* 112-124.
15. Gamberg, C.G. and Hakomori, S.I. (1973) *J. Biol. Chem., 248:* 4311-4317.
16. Hatton, M.W.C. and Regoeczi, E. (1976) *Biochim. Biophys. Acta., 438:* 339-346.
17. Phillips, D.R. (1972) *Biochemistry, 10:* 4582-4587.
18. Laemmli, U.K. (1970) *Nature (Lond.), 227:* 680-685.
19. Bonner, W.A. and Laskey, R.A. (1974) *Eur. J. Biochem., 46:* 83-88.
20. Phillips, D.R. (1974) *Thromb. Diath. Haemorrh., 32:* 207-215.
21. Massini, P. and Lüscher, E.F. (1971)*Thromb. Diath. Haemorrh., 25:* 13-21.
22. Steck, T.L. and Dawson, G. (1974) *J. Biol. Chem., 249:* 2135-2142.
23. Davey, M.G. and Lüscher, E.F. (1968) *Biochim. Biophys. Acta., 165:* 490-506.
24. Tuech, J.K. and Morrison, M. (1974) *Biochem. Biophys. Res. Commun., 59:* 352-360.
25. Phillips, D.R. and Agin, P.P. (1975) "Platelets", Ulutin, O.N., Ed., American-Elsevier Publishing Co., Amsterdam, pp. 132-138.
26. Detwiler, T.C. and Feinman, R.D. (1973) *Biochemistry, 12:* 282-289.
27. Tollefsen, D.M., Feagler, J.R. and Majerus, P.W. (1974) *J. Biol. Chem., 249:* 2646-2651.